Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies

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Abstract □ This is a summary report of the conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies." The conference was held from December 3 to 5, 1990, in the Washington, D.C., area and was sponsored by the American Association of Pharmaceutical Scientists, the U.S. Food and Drug Administration, Federation International Pharmaceutique, Health Protection Branch (Canada), and the Association of Official Analytical Chemists. The report presents our assessment of the major agreements and issues discussed at the conference. The report is also intended to provide guiding principles for validation of analytical methods used in bioavailability, bioequivalence, and pharmacokinetics studies in humans and animals. The objectives of the conference were as follows: (1) to reach a consensus on what should be required in analytical methods validation and the procedures to establish validation; (2) to determine processes of application of the validation procedures in bioavailability, bioequivalence, and pharmacokinetics studies; and (3) to develop a report on analytical methods validation that may be referred to in developing future formal guidelines. Acceptable standards for documenting and validating analytical methods with regard to processes, parameters, or data treatments are discussed because of their importance in assessing pharmacokinetic, bioavailability, and bioequivalence studies. Other topics that were considered essential in the conduct of pharmacokinetic studies or in establishing bioequivalency criteria, including measurement of drug metabolites and stereoselective determinations, are also discussed.

Analytical methods that are used for the quantitative determination of drugs and their metabolites in biological samples play a significant role in evaluation and interpretation of bioavailability, bioequivalence, and pharmacokinetic data. It is essential to use well-characterized and fully validated analytical methods to yield reliable results that can be satisfactorily interpreted. Analytical methods and techniques are constantly being changed and improved; in many instances, these methods are at the cutting edge of the technology. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from drug to drug. Moreover, the appropriateness of the technique may be influenced by the ultimate objective of the study. Specific validation criteria are needed for methods intended for analysis of each analyte (drug and/or metabolite). Although validation of each method will be independent of those of other methods, there may be situations in which comparison of the methods will be necessary (e.g., when more than one method has been used in a long-term study). When sample analysis is conducted at more than one site, it is necessary to validate the analytical method(s) at each site and provide appropriate validation information for different sites to establish interlaboratory reliability. Unless a method is used on a regular basis, providing confidence in its continued validity, it is essential to document that the method is still valid before analysis of samples in the study. Adequate validation for methods not used on a regular basis often consists of running a standard curve with new quality-control samples to show that the responses, relationship, and general characteristics of the method are similar to previous validation results.

Validation of Analytical Methods

Method validation includes all of the procedures required to demonstrate that a particular method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. Some of the more commonly used bioanalytical techniques include (1) chemical methods [e.g., chromatography (GC and HPLC) and various procedures with MS (direct MS, MS–MS, and combination techniques such as GC–MS and LC–MS)] and (2) biological methods [e.g., those based on immunoassay procedures (RIA, enzyme-multiplied immunoassay technique [EMIT], enzyme-linked immunosorbent assay [ELISA]), and microbiological methods)]. Many of the principles, procedures, and requirements are common to all types of analytical methods.

The parameters essential to ensure the acceptability of the performance of an analytical method are stability of the drug in the matrix under the storage conditions of the study, accuracy, precision, sensitivity, specificity (selectivity), response function, and reproducibility. Although there are various stages in the development and validation of an analytical procedure, the validation of the analytical method can be envisaged to consist of two distinct phases: (1) the development phase, in which the assay is defined, and (2) the application phase, in which the method is applied to actual analysis of samples from pharmacokinetic, bioavailability, and bioequivalence studies.

Development of Analytical Methods: Chemical Assays—The following principles of validation of analytical methods provide steps for developing a new method or establishing an existing method in a particular laboratory for the first time. Any modification of an analytical method would require revalidation of the procedures. Validation of analytical methods should be performed to support pharmacokinetic,

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bioequivalence, and related studies in a new drug application (NDA) or an abbreviated new drug application (ANDA). Full validation of methods may not be necessary in conducting exploratory pharmacokinetic studies. It is suggested that validation include investigation of samples from dosed subjects.

Principles for Establishing a Valid Method—The following principles should be used to establish a valid method.

(1) A specific, detailed description and protocol of the method should be written (standard operating procedure).

(2) Each step in the method should be investigated to determine the extent to which environmental, matrix, material, or procedural variables, from the time of collection of the material up to the time of analysis and including the time of analysis, may affect the estimation of analyte in the matrix. Variability of the matrix due to its physiological state may need to be considered.

(3) A method should be validated for the intended use with an acceptable protocol. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).

(4) Whenever possible, the same biological matrix as that in the intended samples should be used for validation purposes. (For tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices may suffice.) The stability of the analyte (drug and/or metabolite) in the matrix during the collection process and the sample storage period should be assessed, preferably before sample analysis. It is recommended that the stability of the analyte in the matrix from dosed subjects be confirmed. Accuracy, precision, reproducibility, response function, and the specificity of the method with respect to endogenous substances, metabolite(s), and known degradation products should be established with reference to the biological matrix. In regard to specificity, there should be evidence that the substance being quantitated is the intended analyte.

(5) The concentration range over which the analyte will be determined must be defined in the method, on the basis of evaluation of actual standard samples over the range (including their statistical variation). This procedure defines the standard curve.

(6) It is necessary to use a sufficient number of standards to define adequately the relationship between concentration and response. The relationship between response and concentration must be demonstrated to be continuous and reproducible. The number of standards to be used will be a function of the dynamic range and the nature of the concentration–response relationship. In many cases, five to eight concentrations (excluding blank values) may define the standard curve. More standard concentrations may be necessary for nonlinear relationships than would be for linear relationships.

(7) The accuracy and precision with which known concentrations of analyte in the biological matrix can be determined must be demonstrated. Within- and between-run accuracy and precision should be calculated with commonly accepted statistical procedures. Determination of accuracy and precision can be accomplished by analysis of replicate sets of analyte samples of known concentrations from an equivalent biological matrix. At least three concentrations representing the entire range of the calibration curve should be studied: one near the lower limit of quantitation (LOQ), one near the center, and one near the upper boundary of the standard curve. For a method to be considered valid, specific criteria must be set for accuracy and precision over the range of the standard curve.

(8) The LOQ is the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision, and variability. The LOQ should be determined by using at least five samples independent of standards and by determining the coefficient of variation and/or appropriate confidence interval. The LOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection (LOD; see glossary).

Specific Recommendations for Method Validation—The following recommendations should be considered when validating a method.

(1) The stability of the analyte in the biological matrix at the intended storage temperature(s) should be established. In addition, the influence of freeze–thaw cycles (a minimum of two cycles at two concentrations in duplicate) should be studied.

(2) The specificity of the assay method should be established with six independent sources of the same matrix.

(3) The accuracy and precision should be determined with a minimum of five (excluding blank sample) determinations per concentration. The mean value should be within ±15% of the actual value, except at LOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% coefficient of variation (CV), except for LOQ, where it should not exceed 20% CV. Other methods of determining accuracy and precision that meet these limits may be equally acceptable.

(4) The standard curve should consist of five to eight standard points, excluding blank, with single or replicate samples. The standard curve should cover the entire range of expected concentrations.

(5) The simplest relationship for response versus concentration (response function) should be determined, and the fit should be statistically tested. The function should be represented with an appropriate algorithm or graphical technique.

Application to Routine Drug Analysis—Many of the principles for establishing a valid method are relevant to prestudy validation. This section will emphasize the validation parameters that should be performed during routine application of a method to a particular study.

In general, with acceptable variability as defined by validation data, analysis of biological samples can be done by single determination without a need for duplicate or replicate analysis. The need for duplicate analysis should be assessed on a case-by-case basis. For example, for a robust procedure of low variability, with accuracy and precision routinely well within tolerances, single analysis would suffice. For a difficult procedure with a labile analyte, when the precision and accuracy tolerances are difficult to achieve, duplicates may be essential. A procedure should be developed that documents the reasons for reanalysis.

A standard curve should be generated for each analytical run for each analyte and should be used to calculate the concentration of analyte in the unknown samples assayed with that run. It is important to use a standard curve that will cover the entire range of concentrations in the unknown samples. Estimation of unknowns by extrapolations of standard curves below the low standard or above the high standard is not recommended. Instead, it is suggested that the standard curve be redetermined or samples be reassayed after dilution. The quality-control (QC) samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.

In summary, (1) a standard curve should consist of five to eight standard points, excluding blank (either single or replicate), covering the entire range; (2) the response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation; and (3) the system suitability is based on the analyte and technique (a specific procedure or sample can be identified to assure the optimum operation of the system employed).

Acceptance Criteria for the Run—*Accuracy and Precision*—The acceptance criteria are not more than 15% CV for precision and not more than 15% deviation from the nominal

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value for accuracy. However, at the LOQ, 20% is acceptable for both precision and accuracy. It is desirable that these tolerances be provided both for intraday and interday or interrun experiments.

QC Samples—QC samples in duplicate at three concentrations (one near the LOQ, one in midrange, and one approaching the high end of the range) should be incorporated into each run. The results of the QC samples provide the basis of accepting or rejecting the run.

At least four of the six QC samples must be within 20% of their respective nominal values; two of the six QC samples (not both at the same concentration) may be outside the ±20% respective nominal value. A confidence-interval approach yielding comparable accuracy and precision is an acceptable alternative.

Repeat Analysis—The protocol for repeat analysis should be established a priori. Some aberrant values can be identified, which can be attributed to processing errors, equipment failure, poor chromatography, or QC samples outside predefined tolerance. Cautious use of "pharmacokinetic fit", such as a double peak, may call for repeat analysis of some samples in the study, but the reasoning should be clearly documented.

Validation of Analytical Methods: Immuno- and Microbiological Assays

Many of the parameters for and principles of analytical validation for chemical methods are also applicable to immuno- and microbiological methods, but there are some specific differences. In immuno- and microbiological assays, the response must be shown to be related to the concentration of the analyte in question.

Selectivity Issues—As with chromatographic methods, it must be demonstrated that the bioassay is selective for the analyte. An alternative method, if rigorously established, may be used to compare the results of the bioassay. For bioassay, an appropriate combination of other techniques may be used to show selectivity, including the following: (1) comparison of standards in biological fluids with standards in buffer to detect matrix effects; (2) parallelism of diluted clinical samples with diluted standards to detect presence of closely related compounds; (3) serial separation techniques (e.g., extraction) and chromatography, with the bioassay as detector, to demonstrate that the response is due only to the analyte in question; and (4) metabolite (or endogenous compound) cross-reaction assessed initially by comparison of displacement curves (in critical cases, metabolite cross-reaction should also be assessed by addition of metabolite to analyte). Similar criteria will be applicable when the drug is concomitantly administered with other drugs.

Quantitation Issues—These issues are summarized as follows:

(1) Criteria for precision and accuracy of immuno- and microbiological assays should be based on the requirements of the study and should match those of chromatographic methods. Any decision to run the sample analysis in single, duplicate, or triplicate should be based on variability.

(2) Immunoassay standard curves are essentially nonlinear and, in general, require more concentration points to define the fit over the range claimed.

(3) It should be established that an acceptable curve-fitting model is being used by examining statistics for goodness of fit and by back-calculating results of standards and control samples.

(4) Both upper and lower LOQ values must be defined by acceptable accuracy, precision, or confidence-interval criteria based on the study requirements.

(5) For all assays, the key factor is the accuracy of the reported results. This accuracy may be improved by use of replicate samples. When replicate samples need to be measured during validation to improve accuracy, the same procedure must be followed for unknown samples.

(6) If there are intermediate steps between the plasma (or other biological matrices) and the final assay (such as extraction of biological sample followed by immunoassay) and if parallel processed standards in the biological matrix are not being used, it is necessary to establish recovery and use it in determining results. Possible approaches to assess efficiency and reproducibility of recovery are use of radiolabeled tracer analyte (quantity too small to affect the assay), advance establishment of reproducible recovery, and use of an internal standard that is not recognized by the antibody but can be measured by another technique.

(7) Correction for nonspecific matrix effects can be accomplished with separation techniques that remove the effect or the matrix. These techniques may be used in defining the standard curve for both controls and samples. The use of standards in the matrix is recommended. This approach will obviate many of the earlier mentioned concerns.

Other Issues—*Commercial Kits*—Commercial kits are available for both immuno- and microbiological assays, and the analytical methods based on such kits should be validated. The validation assures that the bioassay kit is applicable to the study problem and that subsequent batches or lots of kits have performance characteristics similar to the original validated kit or test. Any modifications and extensions of assays from one kit (or test) to another must be validated.

Measurement of Metabolite(s)—The complex area of determination of drug metabolites in bioavailability studies to support drug submissions was discussed in the conference. The questions differed somewhat according to the objective of the application of the bioanalytical measurement (e.g., bioequivalence versus pharmacokinetic profiling). Some situations exist in studies of bioavailability and bioequivalence in which (1) the parent drug cannot be measured in biological samples and only the metabolite can be measured, (2) the parent drug and active and/or inactive major metabolite(s) can be measured, (3) more than one metabolite is present, or (4) the accumulation of metabolite is augmented (e.g., in the case of renal impairment). Under such situations, should one measure the metabolite(s)? Can decision criteria be developed for measuring the metabolite in such situations? From the discussions, the following suggestions were made:

(1) All methods applied for measuring drug and metabolite(s) should be validated for that particular study matrix, with the same general parameters mentioned earlier (accuracy, precision, specificity, recovery, and reproducibility).

(2) Pharmacokinetic, bioavailability, and bioequivalence studies should be based on the moieties that contribute significantly to the pharmacologic or therapeutic effect.

Assay of Stereoisomers—The need for stereoselective determination in bioavailability and bioequivalence studies was another issue that was discussed. There are many drugs that are administered as racemic mixtures, and they may undergo stereoselective metabolism and/or elimination. One isomer may be more active than the other. Under what circumstances should one measure individual drug isomers and/or metabolite(s) isomers from the biological matrix? The following suggestions were made: (1) all methods used for measurement of stereoisomer should be validated (with emphasis on stereospecificity); (2) for bioequivalence studies of an existing racemic product, a stereospecific assay is not required if the rate and extent profiles are superimposable (within usual statistical boundaries); and (3) for new chemical entities, the pharmacokinetic profiles for the stereoisomer should be characterized in normal subjects.

Pharmacodynamic Measurements—The final difficult issue identified was the area of pharmacodynamic measurements.

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The suggestions were as follows. (1) all pharmacodynamic procedures used for definitive bioequivalence or related studies must be fully validated under controlled conditions and should include a placebo and (2) the pharmacodynamic effect measured for bioequivalence studies should be related to the actual pharmacologic (therapeutic) end point of the activity of the drug.

Glossary

Accuracy: closeness of determined value to the true value. Generally, recovery of added analyte over an appropriate range of concentrations is taken as an indication of accuracy. Whenever possible, the concentration range chosen should bracket the concentration of interest.

Analyte: a specific, unique chemical moiety in the form(s) it would be found in a biologic matrix.

Biological matrix: a unique material of biological origin that can be prepared in a reproducible manner (e.g., blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues).

Limit of detection (LOD): the lowest concentration of an analyte that the analytical process can reliably differentiate from background levels.

Limit of quantitation (LOQ): the lowest concentration of an analyte that can be measured with a stated level of confidence.

Linear range: generally taken as the range over which the procedure has been demonstrated to give a linear detector response. A reproducible, nonlinear response curve, however, can also be acceptable (nonlinearity is certainly the case with immunological procedures).

Method: a set of all of the procedures involved in the collection, processing, storage, and analysis of a biological matrix for an analyte.

Precision: the closeness of replicate determinations of an analyte by an assay. Precision can be further subdivided into within-day or intra-assay precision and between-day or inter-assay precision.

Specificity: the ability of method to measure only what it is intended to measure.

Standard curve: the relationship between the experimental response value and the analytical concentration; also referred to as a calibration curve.

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